

This is the peer reviewed version of the following article:

*Identification of differentially expressed small RNAs and prediction of target genes in Italian Large White pigs with divergent backfat deposition*

Davoli, R.; Gaffo, E.; Zappaterra, M.; Bortoluzzi, S.; Zambonelli, P.

Animal Genetics 2018 volume 49 Issue 3 pages 205-214

which has been published in final form at <http://dx.doi.org/10.1111/age.12646>

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

**Identification of differentially expressed small RNAs and prediction of target genes in Italian Large White pigs with divergent backfat deposition**

Running head: MiRNA-gene interaction in pig backfat

Roberta Davoli<sup>1\*</sup>, Enrico Gaffo<sup>2\*</sup>, Martina Zappaterra<sup>1</sup>, Stefania Bortoluzzi<sup>2§</sup>, Paolo Zambonelli<sup>1§§</sup>

<sup>1</sup> Department of Agricultural and-Food Sciences (DISTAL), University of Bologna, Viale G. Fanin 46, 40127 Bologna, Italy.

<sup>2</sup> Department of Molecular Medicine, University of Padova, Viale G. Colombo 3, 35131 Padova, Italy

\* Equally contributing first Author

§ Equally contributing last Author

§To whom correspondence should be addressed: [paolo.zambonelli@unibo.it](mailto:paolo.zambonelli@unibo.it)

## Summary

The identification of the molecular mechanisms regulating pathways associated to the potential of fat deposition in pigs can lead to the detection of key genes and markers for the genetic improvement of fat traits. MicroRNAs (miRNAs) interactions with target RNAs regulate gene expression and modulate pathway activation in cells and tissues. In pigs, miRNA discovery is well far from saturation and the knowledge of miRNA expression in backfat tissue and particularly of the impact of miRNA variations are still fragmentary. We characterized by RNA-seq the small RNAs (sRNAs) expression profiles in Italian Large White pig backfat tissue. Comparing two groups of pigs divergent for backfat deposition, we detected 31 significant differentially expressed (DE) sRNAs, 14 up-regulated (including ssc-miR-132, ssc-miR-146b, ssc-miR-221-5p, ssc-miR-365-5p, and the moRNA ssc-moR-21-5p) and 17 down-regulated (including ssc-miR-136, ssc-miR-195, ssc-miR-199a-5p, and ssc-miR-335). To understand the biological impact of the observed miRNA expression variations, we used the expression correlation of DE miRNA target transcripts expressed in the same samples to define a regulatory network of 193 interactions between DE miRNAs and 40 DE target transcripts showing opposite expression profiles and being involved in specific pathways. Several miRNAs and mRNAs in the network resulted to be expressed from backfat related pig QTLs. These results are informative on the complex mechanisms influencing fat traits, shed light on a new aspect of the genetic regulation of fat deposition in pigs, and facilitate the perspective implementation of innovative strategies of pig genetic improvement based on genomic markers.

**Keywords:** swine, backfat, microRNA, moRNA, differential expression, regulatory network,  
genetic improvement

## **Introduction**

Backfat deposition in pigs is an important selection trait closely connected with carcass and meat quality. MicroRNAs (miRNAs) and miRNA-like short RNAs (sRNAs) represent a functionally relevant RNA category modulating the expression of coding messenger RNAs (mRNAs) and noncoding transcripts in almost all tissues. In pigs, only 411 mature miRNAs are included in the current miRBase release 21 (Kozomara and Griffiths-Jones, 2013), being less than 16% of the number of human miRNAs, indicating that miRNA discovery in pigs is far from reaching saturation. To date, only few papers describing miRNAs expression in porcine adipose tissues have been published (Li et al., 2011; Chen et al., 2012; Li et al., 2012; Bai et al., 2014). Recently, the transcription profiles of sRNAs in backfat of two Italian Large White pigs have been described in Gaffo et al. (2014), reporting new miRNAs, isomiRs and moRNAs, and showing the complexity of sRNA population in the porcine backfat tissue. RNA-seq was recently used to study transcriptome variations in backfat tissue comparing two groups of Italian Large White pigs divergent for backfat deposition, getting a deeper knowledge of the molecular processes regulating the potential of fat deposition in pigs (Zambonelli et al., 2016), that is essential to identify key genes and markers for the genetic improvement of fat traits.

In this study, we used RNA-seq to describe the transcription profile of sRNAs expressed in porcine backfat tissue and to identify sRNAs differentially expressed between fat and lean pigs. Furthermore, linking miRNAs and target transcripts whose expression vary in relation to backfat deposition we reconstructed a regulatory network that disclose the impact of miRNAs regulatory action on fat traits.

## **Materials and Methods**

### **Samples collection and sequencing**

For this analysis we used 18 of the Italian Large White pigs and the backfat samples collected and described in Zambonelli et al. (2016). All animals used in this study were kept according to Italian and European laws for pig production and all the adopted procedures were fully compliant with national and European Union regulations for animal care and slaughtering. We considered short RNA-seq data of backfat tissue in 18 animals, comprising 9 pigs with high backfat thickness (FAT) and 9 pigs with low backfat thickness (LEAN), as described before in Zambonelli et al. (2016). The 18 RNA samples were newly sequenced (series record GSE108829) as detailed below.

Backfat samples stored at -80 °C in a deep freezer were used for total RNA extraction using Trizol (Invitrogen) according to the manufacturer's instructions. Extracted RNA was quantified using the Nanodrop ND-1000 spectrophotometer, and the quality of the extracted RNA was assayed using an Agilent 2100 BioAnalyzer (Supplementary Table 1). Eighteen small RNA libraries were prepared from total RNA using the TruSeq Small RNA kit (Illumina) and version 3

of the reagents following the manufacturer's suggested protocol. The libraries were individually tagged and run on 9 lanes of an Illumina GAI.

## Computational analysis of small RNA sequencing data

The bioinformatics pipeline used is summarized in Supplementary Figure 1, informing also on the study design. RNA-seq data were processed with a combination of the miR&moRe pipeline (Bortoluzzi et al., 2012; Gaffo et al., 2014) and of miRDeep2 (Friedländer et al., 2011), which was exploited to discover new precursor hairpins that express mature miRNAs (NPmiRNAs) in the samples: we run miR&moRe on the known porcine miRNAs plus a high confidence set of predictions, obtained by miRDeep2.

MiRDeep2 v0.0.5 was run with default parameters pooling the sample read sets. Input genome and miRNA annotation were the same used for miR&moRe (UCSC database susScr3 genome and miRBase release 21). We filtered miRDeep2 predictions restricting to precursors with miRDeep2 score greater than 1.0, length greater than or equal to 50 nt and predicted probability of being a miRNA greater than 60% (Londin et al., 2015). The novel precursor annotation predicted by miRDeep2 was added to miRBase swine miRNA annotation and given as input to miR&moRe, which was run on the 18 newly sequenced samples.

MiR&moRe quantifies small RNAs from RNA-seq experiments, detect as well miRNA isoforms (isomiRs), novel miRNAs expressed in known hairpin precursors with only one miRNA annotated, and identify also miRNA-offset RNAs (moRNAs; Bortoluzzi et al., 2011; Bortoluzzi et al., 2012; Guglielmelli et al., 2015). The miR&moRe pipeline performs a preliminary cleaning and quality preprocessing of the input raw sequences. Reads passing the quality filter are

aligned to the reference miRNA precursors and to the reference genome for expression quantification. Identification and expression quantification of isomiRs and moRNAs follow from the alignments and sequence folding predictions. Small RNAs expression levels are measured as read alignment counts in each sample.

## Differential expression assessment and validation

Read counts were normalized across all the samples according to the DESeq2 (v1.4.5; Love et al., 2014) approach. Small RNAs represented by less than ten normalized reads considering all samples were excluded from further analysis. Short RNA differential expression comparing FAT and LEAN groups was assessed by DESeq2, considering significant those variations with FDR < 0.05 (Benjamini-Hochberg adjusted P-values).

Validations of miRNAs differentially expressed according to RNA-seq analysis have been obtained by Quantitative Real Time PCR (qRT-PCR) analysis on the same samples, performed in triplicate. The TaqMan® Micro RNA Assay kit (Applied Biosystems) was used, following the customer protocol, as reported in Gaffo et al. (2014), and using as internal reference the U6 snRNA (Chen et al., 2012; Yu et al., 2012). For the relative miRNA quantification, the PCR-derived cycle threshold (Cq) of target miRNAs is compared with that of a stably expressed endogenous miRNA from the same sample. The difference between these values is the  $\Delta Cq$  value (Rai et al., 2012). A t-test was used to assess the significance of the differential expression estimated by qRT-PCR, considering a significance threshold of P-value < 0.05.

## Small RNA target prediction on backfat long RNA data

The transcription profiles of long RNAs in the same pig backfat samples (GSE68007) already analyzed in Zambonelli et al. (2016) were used for integrative analyses with miRNA expression profiles, considering matched miRNA and transcript expression data in backfat of FAT and LEAN animals. MiRanda v3.3a (Enright et al., 2003) was applied to predict target sites on the 63,418 transcript sequences reported by Zambonelli et al. (2016). For each differentially expressed miRNA (DEM), the prediction was computed using the most abundant isomiR sequences (detected as reported in Gaffo et al., 2014), keeping only isomiRs contributing each at least 10% of the miRNA expression.

Next, focusing on differentially expressed small RNAs, we calculated Spearman pairwise correlations between sRNA and DEM expression profiles, and tested the significance of the correlations. According to largely prevalent repressive role of miRNAs on target transcripts, we focused on the negative correlations, whose association P-values were corrected for multiple tests (Benjamini-Hochberg). Only negative correlations with FDR at most 10% were considered significant to identify miRNA-transcript relations that are supported by expression data analysis. Significant miRNA-transcript relations with correlation  $<-0.6$  were selected to draw an interaction network, using Cytoscape version 3.3. Pathways enriched in genes included in the network have been calculated with EnrichR (<http://amp.pharm.mssm.edu/Enrichr/>; Kuleshov et al., 2016), selecting KEGG and Reactome databases and considering significant those pathways with adjusted P-value at most 0.1 and including at least two genes.

Quantitative Trait Loci enrichment on target genomic regions



For each pig QTL annotated in the PigQTL database ([http://www.animalgenome.org/cgi-bin/QTLdb/SS/download?file=gbpSS\\_10.2](http://www.animalgenome.org/cgi-bin/QTLdb/SS/download?file=gbpSS_10.2)), we counted the number of differentially expressed sRNAs and their predicted target genes considering only those with differentially expressed transcripts with FDR at most 30% according to Zambonelli et al. (2016) mapping in the genomic region. QTL enrichment in miRNA and genes was tested using the upper-tailed hypergeometric test for over-representation, with one side mid p-values as defined in Rivals et al. (2007). The analysis used as background the pig genome annotation (v 10.2.80) merged with the new genes discovered in Zambonelli et al. (2016), resulting in 34,617 genes, and the new sRNAs detected in this study. We considered as significantly enriched only those QTLs showing adjusted P-values <0.1.

## Results

### Small RNA identification and quantification from RNA-seq data

RNA-seq analysis produced in 97 million reads per sample on average, that after read trimming and subsequent filtering steps (Supplementary Table 2) were reduced to 37 million high quality reads per sample, in average, that were further processed for the sRNAs characterization. We applied stringent filtering criteria in the processing of the raw data to obtain high quality read sets deprived of sequencing artifacts, at the cost of high number of discarded sequences. This approach and the number of biological replicates contributed to the reliability of downstream predictions, in particular regarding novel elements like moRNAs and isomiRs.

MiRDeep2 analysis of RNA-seq data predicted 1,340 new miRNA precursors characteristic of pig genome, expressing 2,680 putative novel mature miRNAs, whose sequences do not overlap each other and with different sequence from pig miRNA precursors reported in miRBase. Of these, 103 NPmiRNAs passed the filtering criteria and were considered for following analyses. Overall, 426 sRNAs resulted expressed in the new dataset, including 231 known miRNAs, 69 new miRNAs from known precursors, 103 new miRNAs from new precursors, and 23 moRNAs (Supplementary Table 3).

As observed in Gaffo et al. (2014), the expression distribution was very skewed: only 24 known miRNAs (5.6% of the expressed sRNAs) accounted for the 90% of the total expression, and only 21.1% of expressed sRNAs accounted the 99% of the total expression (Supplementary Figure 2). Ssc-miR-10b, ssc-miR-143-3p and ssc-148a-3p resulted very abundant and accounted together for about the 52% of the total expression, with the first two accounting for respectively about 33% and 13% of the expression.

## Differentially expressed sRNAs

Among the 426 sRNAs expressed in the considered porcine backfat samples, 31 resulted differentially expressed comparing LEAN and FAT animals (Table 1, Supplementary Table 4): 18 known miRNAs, 6 new sister miRNAs, 6 miRNAs from new precursors and ssc-moR-21-5p, whose existence was previously validated by qRT-PCR (Gaffo et al., 2014). Figure 1 shows the unsupervised clustering and heatmap of expression profiles of the 31 DEM in the 18 samples and two animal groups considered. SRNAs up- and down-regulated in FAT animals were 14 and

17, respectively. Up-regulated sRNAs include six known miRNAs (ssc-miR-146b, ssc-miR-365-5p, ssc-miR-221-5p, ssc-miR-222, ssc-miR-132 and ssc-miR-1306-5p) the moRNA ssc-moR-21-5p, a new pig sister miRNA (ssc-miR-128-1-5p) and six mature miRNAs from newly predicted precursors. All down-regulated sRNAs are miRNAs from already annotated precursors, including 12 known miRNAs (ssc-let-7c, ssc-miR-130a, ssc-miR-181c, ssc-miR-199a-5p, ssc-miR-199a-3p, ssc-miR-199b-3p, ssc-miR-195, ssc-miR-193a-3p, ssc-miR-335, ssc-miR-133a-3p, ssc-miR-545-3p, ssc-miR-136) and 5 new sister miRNAs (ssc-miR-214-5p, ssc-miR-99a-3p, ssc-miR-101-2-5p, ssc-miR-136-3p, ssc-miR-362-3p, ssc-miR-128-1-5p).

We selected 9 DEMs, 8 miRNAs and ssc-moR-21-5p, for the qRT-PCR validation of differential expression. A good agreement between RNA-seq and qRT-PCR expression estimates was observed ( $R^2=0.78$ , Figure 2A) supporting the robustness of the data reported for all the small RNAs detected in this study. Indeed, all the comparisons performed show the same trend between the two analyses, and 5 out of 9 DE sRNAs resulted significantly differentially expressed also according to RT-PCR estimations (Figure 2B).

## IsomiR composition of differentially expressed sRNAs

IsomiRs were investigated for the DEM showing more than one variant (18 known and 6 novel-precursor miRNAs out of the 31 DEM). Fifty-nine distinct isomiRs were detected, specifically 58 in LEAN and 55 in FAT of which 54 were in common. Four isomiRs are specific for LEAN samples and one is specific for FAT samples (Supplementary Table 5).

The number of isomiRs contributing at least 10% of each DEM expression ranged from one to four, a maximum value detected in three cases (chr5\_31322-3p, JH118494-1\_44794-3p,

chr9\_40038-5p). Three miRNAs (chr7\_37486-3p, ssc-miR-136 and ssc-miR-193a-3p) are represented by only one major isoform accounting for more than half of the miRNA expression (Supplementary Figure 3A). Most isoforms, according to miR&moRe classification, are variations of the length: isomiRs in the “shorter\_or\_longer” category are 32 out of 58 in LEAN and 29 out of 55 in FAT; the variants perfectly matching the reference sequence (“exact” variants, or “canonical” isomiRs) are 18 in both groups; and “one-mismatch” variation isomiRs are eight. Less than half of DEMs (10 cases in LEAN and 11 in FAT) express the canonical isomiR as the major form. Conversely, “shorter or longer” isoforms compose the largest part of the expression in 12 LEAN and 11 FAT cases. Notably, in 6 DEMs the exact isomiR contributed less than 10% to the miRNA expression (Supplementary Figure 3B). These findings are consistent with our previous results (Gaffo et al., 2014) showing a similar distribution of isomiRs and indicating that in pig backfat the canonical miRNA isoform is not always the most expressed for the miRNA.

## Network of short and long differentially expressed RNA interactions in pig backfat

The target prediction considered 66 sRNA sequences, including 59 abundant isomiRs from the 18 known miRNAs and the 6 NPMiRNAs, plus 6 new sister-miRNAs and ssc-moR-21-5p. The predicted relations with significant strong negative correlations accounted for 56,683 sRNA-transcript interactions, involving 22,362 transcripts from 12,373 unique genes. All the 66 isomiRs potentially targeted at least one transcript each. Among the known miRNAs, ssc-miR-365-5p presented the largest number of targets (3,095 different transcripts targeted, corresponding to 2,624 genes), while in absolute chr3\_26283-5p has the largest number of

target transcripts (3,383; 2,878 genes). Ssc-miR-136-3p has the smallest number of targets (524; 465 genes), while among the new miRNAs chr7\_37486-3p has the minimum (1,011; 862 genes). Considering the very large number of predicted interactions we focused particularly on the predicted sRNA targets included among the 86 DETs reported in Zambonelli et al. (2016), resulting in 193 putative regulatory interactions between 30 DEMs and 40 target DETs (Supplementary Table 6) that may contribute to regulate the metabolic activity of porcine backfat. The network in Figure 3A-B show the interactions supported by strong correlation. KEGG and Reactome pathways significantly enriched in target DETs in the network are shown in Figure 3C. The down regulated genes *HSPA1A*, *HSPA1B*, and *DNAJB1* are linked to cellular stress responses, to the regulation of stress-induced transcription by *HSF1*, and its modulation by attenuation that occurs during continuous exposure to intermediate heat shock conditions or upon recovery from stress. The same three genes and *MRC1* and *ATP6V0D2* participate in several pathways linked to immunity. Furthermore, five genes (*ADSSL1*, *AKAP5*, *BCAT1*, *HMOX1*, and *PLIN2*) are linked to metabolic pathways. The involvement of several genes in the network in specific pathways is informative on the putative regulatory activity of selected miRNAs beyond single targets, suggesting their impact on pathway regulation by coordinated activity on several functionally connected genes. Four of the metabolism-linked genes are overexpressed in FAT animals and resulted under putative control of common underexpressed miRNAs, with both *BCAT1* and *AKAP5* possibly repressed by the ssc-let-7c, and *HMOX1* and *BCAT1* by ssc-miR-195. The underexpressed stress responses genes are under putative control of specific miRNAs, as *HSPA1A*, *HSPA1B*, and *DNAJB1* can be

coordinately repressed by the upregulated and validated ssc-miR-146, and the triplet *HSPA1B*, *DNAJB1* and *HSP70* by ssc-miR-365.

## QTL enrichment of differentially expressed sRNAs and mRNAs loci

As miRNAs and genes with transcripts differentially expressed in LEAN and FAT animals and their interactions could be implicated in genetic modulation of backfat deposition, we looked for the overlap between their loci and pig QTL, and we observed 56 significantly enriched QTL (Supplementary Table 7). Notably, a significant enrichment in miRNAs and their target DE genes was observed for four backfat-specific carcass quality QTLs (Supplementary Table 7). Two largely overlapping QTLs (QTL 21222 “Average backfat thickness” chr4:74,725,606-82,527,275; QTL 21228 “Backfat at first rib” chr4: 76,741,953-83,693,913) covering approximately 9 Mb contains four DE transcripts from *PENK* gene and from a predicted gene. Two enriched “Average backfat thickness” QTLs were identified in chromosome 8 (QTL 29568, chr8: 33,033,004-33,985,796, XLOC\_032101 / *UCHL1*) and 12 (QTL 5990, chr12: 23,672,762-60,040,247). The most interesting enriched QTL is located in chromosome 12 and contains the genes expressing eight DETs, and notably five DE miRNAs (the up-regulated ssc-miR-193a-3p and ssc-miR-195 and the down-regulated ssc-miR-132, ssc-miR-365 and ssc-moR-21-5p) strengthening the indication of a possible role of these sRNAs in the backfat phenotype variation.

## Discussion

272 We reported the landscape of sRNA expression in swine backfat, detecting and quantifying 426  
273 sRNAs expressed, including 403 miRNAs and 23 moRNAs, confirming previous data (Gaffo et al.,  
274 2014) and extending them, by increasing the number of sRNAs detected in pig backfat.

275 The FAT and LEAN animals expression profile comparison disclosed 31 differentially expressed  
276 sRNAs whose abundance is related to backfat level, including 30 miRNAs and a moRNA, ssc-  
277 moR-21-5p, that resulted downregulated in FAT animals. These results are backed up by  
278 quantitative real-time PCR validations conducted on a sizeable subset of the DE sRNAs detected  
279 by RNA-seq (Figure 2), including known miRNAs, new mature miRNAs of known precursors, and  
280 a moRNA, with different expression levels and variable variations strengths in the comparison  
281 between FAT and LEAN animals.

282 Thirthy DE miRNAs possibly targeted 40 out of 86 backfat DETs, while no putative targets were  
283 detected among the same backfat DETs for ssc-moR-21-5p. This study confirmed a possible link  
284 between ssc-moR-21 and adipocyte physiology (Gaffo et al., 2014) that deserves further  
285 investigation.

286 Altogether, almost two thousands putative miRNA-target interactions emerged considering the  
287 correlation between the DE miRNAs and the DE mRNAs and represented as a functional  
288 network (Figure 3).

289 The majority of the transcripts included in the network are coding (green squares) while there  
290 are four putative noncoding (grey and orange squares). *BCAT1* (branched chain amino acid  
291 transaminase 1) and *GPNUMB* (glycoprotein Nmb) are represented by both coding and one  
292 noncoding isoforms.

293 The less expressed ssc-miR-199a-5p potentially regulate several transcripts, including coding  
 294 transcripts of different genes, different isoforms derived from the same gene, and unannotated  
 295 transcripts found overexpressed in FAT vs LEAN pigs. As already described in Zambonelli et al.  
 296 (2016) some of them are involved in lipid metabolism (perilipin 2, *PLIN2*) or in the regulation of  
 297 food intake (*GPNMB*). Ssc-miR-199a-5p was found under-expressed in the FAT pigs, in  
 298 agreement with previous data on the underexpression of this miRNA in attenuating cell  
 299 proliferation and promoting lipid deposition in porcine pre-adipocytes (Shi X-E et al., 2014).  
 300 Ssc-miR136, ssc-miR-99a-3p, and ssc-miR-214-5p were down-regulated in FAT vs LEAN pigs. In  
 301 lambs, miR-136 was differentially expressed in subcutaneous adipose tissue compared with  
 302 perirenal fat (Meale et al., 2014). Guo et al. (2012) linked miR-99a to preadipocyte  
 303 differentiation both in porcine intramuscular and subcutaneous vascular stem cells. The same  
 304 miRNA was twofold more expressed in adult subcutaneous adipose tissue than in juvenile  
 305 adipose tissue in pigs by Li et al. (2011). miR-214 was previously related to adipose-derived  
 306 stem cells differentiation in rats) and resulted up-regulated in rat stem cells isolated from  
 307 subcutaneous fat compared with omentum (Hu et al., 2017).  
 308 The importance of ssc-miR-335 in the regulation of biological and molecular processes  
 309 responsible of different fat deposition between FAT and LEAN animals is supported by previous  
 310 data linking this miRNA to the control of fat traits (Nakanishi et al., 2009; Oger et al., 2014; and  
 311 Zhu et al., 2014). In our network, the down-regulation of miR-335 is linked to *BCAT1*  
 312 overexpression. Under-expressed putative targets of overexpressed miRNAs include one  
 313 protein coding gene transcripts (*DNAJB1*) and noncoding transcripts, mostly belonging to heat  
 314 shock protein genes (*HSP70*, *HSPA1A*, *HSPA1B*) that deserve further investigation.



315 Ssc-miR-146b could control the expression of three genes down-regulated all coding for heat  
316 shock proteins (*DNAJB1*, *HSPA1A*, *HSPA1B*), whereas ssc-miR-365-5p was linked to *DNAJB1*,  
317 *HSPA1A* and *HSP70*, indicating that the two overexpressed *miRNAs* could contribute to  
318 regulation pattern of common targets possibly with a synergistic effect. The action of miR-146b  
319 on tissue inflammation was described in human obesity by different Authors (Hulsmans et al.,  
320 2012; Shi C et al., 2014) who reported that, despite the obese condition, an attenuation of  
321 cytokine signaling reduced the effect of the inflammation on the tissues. In visceral and  
322 subcutaneous adipose tissue of over weighted and obese human subjects, miR-146b  
323 overexpression promoted preadipocyte differentiation, not impacting proliferation (Ahn et al.,  
324 2013; Chen et al., 2014). These evidences suggest a likely explanation of the lower level of  
325 expression on backfat tissue in FAT pigs of heat shock protein DETs as response to a chronic  
326 inflammation (Zambonelli et al., 2016) providing as well a link with miRNA expression and  
327 regulatory activity.

328 Of miRNAs overexpressed in FAT animals, ssc-miR-132 was upregulated in the hypothalamus of  
329 rats fed with a high fat diet (Sangiao-Alvarellos et al., 2014), and in omental fat and plasma of  
330 obese humans (Heneghan et al., 2011), whereas the overexpression of miR-221 was associated  
331 to an increase in adipocytokines expression in mice (Parra et al., 2010) and with body mass  
332 index increase in subcutaneous and abdominal adipose tissue in human Pima Indians (Meerson  
333 et al., 2013) where, intriguingly, the increase of body mass index was associated also with the  
334 under-expression of miR-199a-3p, fully in accordance with our observations.

335 Several elements of the network, are expressed by genomic regions harboring QTL associated  
336 with backfat thickness, supporting the presence and contributing to the identification of

positional and functional candidate genes for fat traits. The SSC12 QTL region arbors both the overexpressed ssc-mir-132 and the under-expressed ssc-mir-195. Within a QTL it is possible to found an epistatic effect of different genetic elements acting on opposite direction in the phenotype determination (Cordell, 2002). However, the action on the phenotype of these two miRNAs could be concordant or even synergistic if they repress the expression of genes whose products have opposite roles in biological processes or signaling pathways.

Collectively, our data and the above discussed literature informed on the involvement of miRNAs in the regulation of genes active in adipose tissue, suggesting that the identified DE miRNAs can modulate several functions related to the adipose tissue development and growth, in particular adipocyte differentiation, adipogenesis, lipid deposition, and obesity in humans. Specific miRNAs might modulate as well heat shock transcript expression, supporting the idea that pigs with an increased potential for fat deposition present some aspects similar to those described in human obesity in which a mild inflammation blocks an excessive harmful effect on a high level of inflammation when a chronic increase of fat tissue as in obesity occurs.

We found new genes, mRNAs and miRNAs that, according to sequence and expression data, could be involved in the complex network of regulation of backfat deposition. Our results explain part of the complex mechanisms influencing fat traits, shedding light on new aspect of the genetic regulation of fat deposition in pigs, and facilitate the perspective implementation of innovative strategies of pig genetic improvement based on genomic markers.

## Acknowledgements

This work was supported by Progetto “AGER - Agroalimentare e ricerca”: Advanced research in genomics and processing technologies for the Italian heavy pig production – Hepiget (Grant N. 2011-0279).

The authors declare that they have no competing interests.

## References

- Ahn, J., Lee, H., Jung, C.H., Jeon, T.I., Ha, T.Y., 2013. MicroRNA-146b Promotes Adipogenesis by Suppressing the SIRT1-FOXO1 Cascade. *EMBO Molecular Medicine* 5, 10: 1602–1612. doi:10.1002/emmm.201302647
- Bai, Y., Huang, J.-M., Liu, G., Zhang, J.B., Wang, J.Y., Liu C.-K., Fang, M.-Y., 2014. A Comprehensive microRNA Expression Profile of the Backfat Tissue from Castrated and Intact Full-Sib Pair Male Pigs. *BMC Genomics* 15, 47. doi:10.1186/1471-2164-15-47
- Bortoluzzi, S., Biasiolo M., Bisognin A., 2011. microRNA-Offset RNAs (moRNAs): By-Product Spectators or Functional Players?. *Trends in Molecular Medicine*. 17, 473-474. doi:10.1016/j.molmed.2011.05.005
- Bortoluzzi, S., Bisognin, A., Biasiolo, M., Guglielmelli, P., Biamonte, F., Norfo, R., Manfredini, R., Vannucchi, A.M., 2012. Characterization and discovery of novel miRNAs and moRNAs in JAK2V617F-mutated SET2 cells. *Blood* 119, e120–e130. doi:10.1182/blood-2011-07-368001
- Chen, C., Deng, B., Qiao, M., Zheng, R., Chai, J., Ding, Y., Peng, J., Jiang, S., 2012. Solexa Sequencing Identification of Conserved and Novel microRNAs in Backfat of Large White and Chinese Meishan Pigs. *PloS One* 7, e31426. doi:10.1371/journal.pone.0031426

379 Chen, L., Dai, Y.-M., Ji, C.-B., Yang, L., Shi, C.-M., Xu, G.-F., Pang, L.-X., Huang, F.-Y., Zhang, C.-M.,  
 380 Guo, X.-R., 2014. MiR-146b Is a Regulator of Human Visceral Preadipocyte Proliferation and  
 381 Differentiation and Its Expression Is Altered in Human Obesity. *Molecular and Cellular*  
 382 *Endocrinology* 393, 65–74. doi:10.1016/j.mce.2014.05.022  
 383 Cordell, H.J., 2002. Epistasis: what it means, what it doesn't mean, and statistical methods to  
 384 detect it in humans. *Human Molecular Genetics* 11,2463-2468.  
 385 Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., Marks, D.S., 2003. MicroRNA targets in  
 386 *Drosophila*. *Genome Biology* 5, R1. doi:10.1186/gb-2003-5-1-r1  
 387 Friedländer, M.R., Mackowiak, S.D., Li, N., Chen, W., Rajewsky, N., 2011. miRDeep2 accurately  
 388 identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucl Acids*  
 389 *Res* 40, 37-52. doi:10.1093/nar/gkr688  
 390 Gaffo, E., Zambonelli, P., Bisognin, A., Bortoluzzi, S., Davoli, R., 2014. miRNome of Italian Large  
 391 White pig subcutaneous fat tissue: new miRNAs, isomiRs and moRNAs. *Anim Genet* 45, 685–  
 392 698. doi:10.1111/age.12192  
 393 Guglielmelli, P., Bisognin, A., Saccoman, C., Mannarelli, C., Coppe, A., Vannucchi, A.M.,  
 394 Bortoluzzi S., 2015. Small RNA Sequencing Uncovers New miRNAs and moRNAs Differentially  
 395 Expressed in Normal and Primary Myelofibrosis CD34+ Cells. *PLoS ONE* 10, e0140445.  
 396 doi:10.1371/journal.pone.0140445  
 397 Guo, Y., Mo, D., Zhang, Y., Zhang, Y., Cong, P., Xiao, S., He, Z., Liu, X., Chen. Y. 2012.  
 398 MicroRNAome Comparison between Intramuscular and Subcutaneous Vascular Stem Cell  
 399 Adipogenesis. *PloS One* 7, e45410. doi:10.1371/journal.pone.0045410

400 Heneghan, H.M., Miller,N., McAnena, O.J., O'Brien, T., Kerin, M.J., 2011. Differential MiRNA  
 401 Expression in Omental Adipose Tissue and in the Circulation of Obese Patients Identifies  
 402 Novel Metabolic Biomarkers. *The Journal of Clinical Endocrinology & Metabolism* 96, E846–  
 403 50. doi:10.1210/jc.2010-2701  
 404 Hu, F., Xu, P., Sun, B., Xiao, Z. 2017. Differences in the MicroRNA Profiles of Subcutaneous  
 405 Adipose-Derived Stem Cells and Omental Adipose-Derived Stem Cells. *Gene* 625, 55–63.  
 406 doi:10.1016/j.gene.2017.05.014  
 407 Hulsmans, M., Van Dooren, E., Mathieu, C., Holvoet, P., 2012. Decrease of miR-146b-5p in  
 408 Monocytes during Obesity Is Associated with Loss of the Anti-Inflammatory but Not Insulin  
 409 Signaling Action of Adiponectin. *PLoS ONE* 7, e32794. doi:10.1371/journal.pone.0032794  
 410 Kozomara, A., Griffiths-Jones, S., 2013. miRBase: annotating high confidence microRNAs using  
 411 deep sequencing data. *Nucleic Acids Research* 42, D68–D73. doi:10.1093/nar/gkt1181  
 412 Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S.,  
 413 Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G., Monteiro, C.D., Gundersen,  
 414 G.W., Ma'ayan, A. 2016. Enrichr: a comprehensive gene set enrichment analysis web server  
 415 2016 update. *Nucleic Acids Research*, 44, Web Server issue, W90–97.  
 416 doi:10.1093/nar/gkw377  
 417 Li, G., Li, Y., Li, X., Ning, X., Li, M., Yang, G., 2011. MicroRNA Identity and Abundance in  
 418 Developing Swine Adipose Tissue as Determined by Solexa Sequencing. *Journal of Cellular*  
 419 *Biochemistry* 112, 1318–1328. doi:10.1002/jcb.23045  
 420 Li, H.-Y., Xi, Q.-Y., Xiong, Y.-Y., Liu, X.-L., Cheng, X., Shu, G., Wang, S.-B., Wang, L.N., Gao, P., Zhu,  
 421 X.T., Jiang, Q.Y., Yuan, L., Zhang, Y.L., 2012. Identification and Comparison of microRNAs

422 from Skeletal Muscle and Adipose Tissues from Two Porcine Breeds. *Animal Genetics* 43, 6:  
 423 704–713. doi:10.1111/j.1365-2052.2012.02332.x  
 424 Londin, E., Loher, P., Telonis, A.G., Quann, K., Clark, P., Jing, Y., Hatzimichael, E., Kirino, Y.,  
 425 Honda, S., Lally, M., Ramratnam, B., Comstock, C.E.S., Knudsen, K.E., Gomella, L., Spaeth,  
 426 G.L., Hark, L., Katz, L.J., Witkiewicz, A., Rostami, A., Jimenez, S.A., Hollingsworth, M.A., Yeh,  
 427 J.J., Shaw, C.A., McKenzie, S.E., Bray, P., Nelson, P.T., Zupo, S., Roosbroeck, K.V., Keating,  
 428 M.J., Calin, G.A., Yeo, C., Jimbo, M., Cozzitorto, J., Brody, J.R., Delgrosso, K., Mattick, J.S.,  
 429 Fortina, P., Rigoutsos, I., 2015. Analysis of 13 cell types reveals evidence for the expression of  
 430 numerous novel primate- and tissue-specific microRNAs. *PNAS* 112, E1106-E1115.  
 431 doi:10.1073/pnas.1420955112  
 432 Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for  
 433 RNA- Seq data with DESeq2. *Genome Biology* 15, 550. doi: 10.1186/s13059-014-0550-8.  
 434 Meale, S.J., Romao, J.M., He, M.L., Chaves, A.V., McAllister, T.A., Guan, L.L., 2014. Effect of Diet  
 435 on microRNA Expression in Ovine Subcutaneous and Visceral Adipose Tissues. *Journal of*  
 436 *Animal Science* 92, 3328–37. doi:10.2527/jas.2014-7710  
 437 Meerson, A., Traurig, M., Ossowski, V., Fleming, J.M., Mullins, M., Baier, L.J., 2013. Human  
 438 Adipose MicroRNA-221 Is Upregulated in Obesity and Affects Fat Metabolism Downstream  
 439 of Leptin and TNF- $\alpha$ . *Diabetologia* 56, 1971–79. doi:10.1007/s00125-013-2950-9  
 440 Nakanishi, N., Nakagawa, Y., Tokushige, N., Aoki, N., Matsuzaka, T., Ishii, K., Yahagi, N.,  
 441 Kobayashi, K., Yatoh, S., Takahashi, A., Suzuki, H., Urayama, O., Yamada, N., Shimano, H.,  
 442 2009. The Up-Regulation of MicroRNA-335 Is Associated with Lipid Metabolism in Liver and

443 White Adipose Tissue of Genetically Obese Mice. *Biochemical and Biophysical Research*  
 444 *Communications* 385, 492–96. doi:10.1016/j.bbrc.2009.05.058  
 445 Oger, F., Gheeraert, C., Mogilenko, D., Benomar, Y., Molendi-Coste, O., Bouchaert, E., Caron, S.  
 446 Dombrowicz, D., Pattou, F., Duez, H., Eeckhoutte, J., Staels, B., Lefebvre, P., 2014. Cell-Specific  
 447 Dysregulation of MicroRNA Expression in Obese White Adipose Tissue. *The Journal of Clinical*  
 448 *Endocrinology & Metabolism* 99, 2821–2833. <https://doi.org/10.1210/jc.2013-4259>  
 449 Parra, P., Serra, F., Palou, A., 2010. Expression of Adipose MicroRNAs Is Sensitive to Dietary  
 450 Conjugated Linoleic Acid Treatment in Mice. *PloS One* 5, e13005.  
 451 doi:10.1371/journal.pone.0013005  
 452 Rai, S.N., Ray, H.E., Yuan, X., Pan, J., Hamid, T., Prabhu, S.D., 2012. Statistical analysis of  
 453 repeated microrna high-throughput data with application to human heart failure: A review  
 454 of methodology. *Open access medical statistics* 2012, 21–31. DOI: 10.2147/OAMS.S27907  
 455 Rivals, I., Personnaz, L., Taing, L., and Potier, M.-C., 2007. Enrichment or depletion of a GO  
 456 category within a class of genes: which test? *Bioinformatics* 23, 401–407. DOI:  
 457 10.1093/bioinformatics/btl633  
 458 Sangiao-Alvarellos, S., Pena-Bello, L., Manfredi-Lozano, M., Tena-Sempere, M., Cordido, F.,  
 459 2014. Perturbation of Hypothalamic MicroRNA Expression Patterns in Male Rats After  
 460 Metabolic Distress: Impact of Obesity and Conditions of Negative Energy Balance.  
 461 *Endocrinology* 155, 1838–1850. <https://doi.org/10.1210/en.2013-1770>.  
 462 Shi, C., Zhu, L., Chen, X., Gu, N., Chen, L., Zhu, L., Yang, L., Pang, L., Guo, X., Ji, C., Zhang, C.,  
 463 2014. IL-6 and TNF- $\alpha$  Induced Obesity-Related Inflammatory Response Through

Transcriptional Regulation of MiR-146b. *Journal of Interferon & Cytokine Research* 34, 342–348. doi:10.1089/jir.2013.0078

Shi, X.-E., Li, Y.-F., Jia, L., Ji, H.-L., Song, Z.-Y., Cheng, J., Wu, G.-F., Song, C.C., Zhang, Q.L., Zhu, J.Y., Yang, G.S. 2014. MicroRNA-199a-5p Affects Porcine Preadipocyte Proliferation and Differentiation. *International Journal of Molecular Sciences* 15, 8526–8538. doi:10.3390/ijms15058526

Yu, X., Zhang, X., Dhakal, I.B., Beggs, M., Kadlubar, S., Luo, D., 2012. Induction of cell proliferation and survival genes by estradiol repressed microRNAs in breast cancer cells. *BMC Cancer* 12, 29. doi: 10.1186/1471-2407-12-29

Zambonelli, P., Gaffo, E., Zappaterra, M., Bortoluzzi S., Davoli, R., 2016. Transcriptional profiling of subcutaneous adipose tissue in Italian Large White pigs divergent for backfat thickness. *Animal Genetics* 47, 306-323. doi: 10.1111/age.12413

Zhu, L., Chen, L., Shi, C.-M., Xu, G.-F., Xu, L.-L., Zhu, L.-L., Guo, X.-R., Ni, Y., Cui, Y., Ji, C., 2014. MiR-335, an Adipogenesis-Related MicroRNA, Is Involved in Adipose Tissue Inflammation. *Cell Biochemistry and Biophysics* 68, 283–290. <https://doi.org/10.1007/s12013-013-9708-3>



## Supporting information

Additional supporting information may be found in the online version of this article.

**Supplementary Tables are included in the file: DEM-DET-**

**Target\_SupplementaryTables\_rev.xlsx**

**Table S1 (ST1).** Samples RIN numbers.

**Table S2 (ST2).** Sample raw reads filtering.

**Table S3 (ST3).** Small RNAs expressed.

**Table S4 (ST4).** List of all DE tests obtained.

**Table S5 (ST5).** IsomiRs specific for FAT or LEAN samples.

**Table S6 (ST6).** Differentially expressed small RNA targets among differentially expressed transcripts.

**Table S7(ST7).** QTL enrichment for differentially expressed sRNAs and target genes.

**Figure S1.** Outline of the computational analysis workflow. Long and short RNA-seq data in the same backfat samples of 10 LEAN and 10 FAT animals have been considered; after detection, discovery and quantification of miRNA and miRNA-like small RNAs, integrative analyses of target prediction and of miRNA and transcript expression profiles were used for miRNA-transcript regulatory network inference.

**File: FigureSF1\_rev.pdf**

**Figure S2.** Cumulative expression of gene expression measures show a similar pattern in the FAT and LEAN groups. The small panel inside the figure corresponds to the region shaded in light blue in the main panel.

**File: FigureSF2\_rev.pdf**

**Figure S3.** IsomiR composition of known and newly predicted miRNAs resulting differentially expressed in backfat tissue of LEAN and FAT animals. Colors and labels are relative to each single bar-chart. The same color may refer to different labels. Portion of the bars not reaching 100% represents isoforms composing each less than 10% of the overall miR expression.

**File: FigureSF3\_rev.pdf**

## Figure captions

**Figure 1.** Heatmap of expression profiles of miRNAs differentially expressed (DEMs) comparing FAT vs LEAN animals.

Rows of the heatmap represent the 31 DEMs, columns correspond to samples. The heatmap cells are colored according to the deviance of the sRNA expression in the sample from the average expression of the sRNA, thus red and blue cells represent respectively expression values higher or lower than mean expression across all samples (white) with color intensity proportional to the difference from the mean, in the regularized logarithmic scale.

**File: Figure1\_rev.pdf**

**Figure 2.** Validation by qRT-PCR of eight DE miRNAs and of the DE moRNA detected by RNA-seq. A) Correlation between fold changes calculated according to RNA-seq (horizontal axis) and qRT-PCR (vertical axis) estimations. Dashed line indicates linear smoothing of the points.  $R_s$  = Spearman's rank correlation coefficient. B) Significance of differential expression tests according to qRT-PCR data. T-test p-value: ns = not significant; \* = <0.05, \*\* <0.01; \*\*\* = <0.001.  $\Delta Cq$  = difference between U6 and the target miRNA PCR-derived cycle thresholds.

**File: Figure2\_rev.pdf**

**Figure 3.** Differentially expressed miRNAs and their putative predicted target transcripts differentially expressed. The network depicts the predicted interactions between: A) miRNAs downregulated in FAT animals and putative target transcripts upregulated in FAT animals; B)

miRNAs upregulated in FAT animals and putative target transcripts downregulated in FAT animals; miRNA-target relations with expression profiles correlation  $< -0.6$  are reported, involving 26 miRNA (blue circle nodes) and 28 transcripts (square nodes, coloured green if coding, lilac if non-coding, and orange if reliably non-coding) for a total of 92 interactions (edges). Edge thickness is inversely proportional to the correlation intensity (the smaller the correlation, the thicker the line). Nodes borders are coloured in red if representing a newly predicted transcript or miRNA precursor, orange for new sister miRNAs, and green for known miRNAs. C) KEGG and Reactome pathways significantly enriched in genes included in the network of panel A-B.

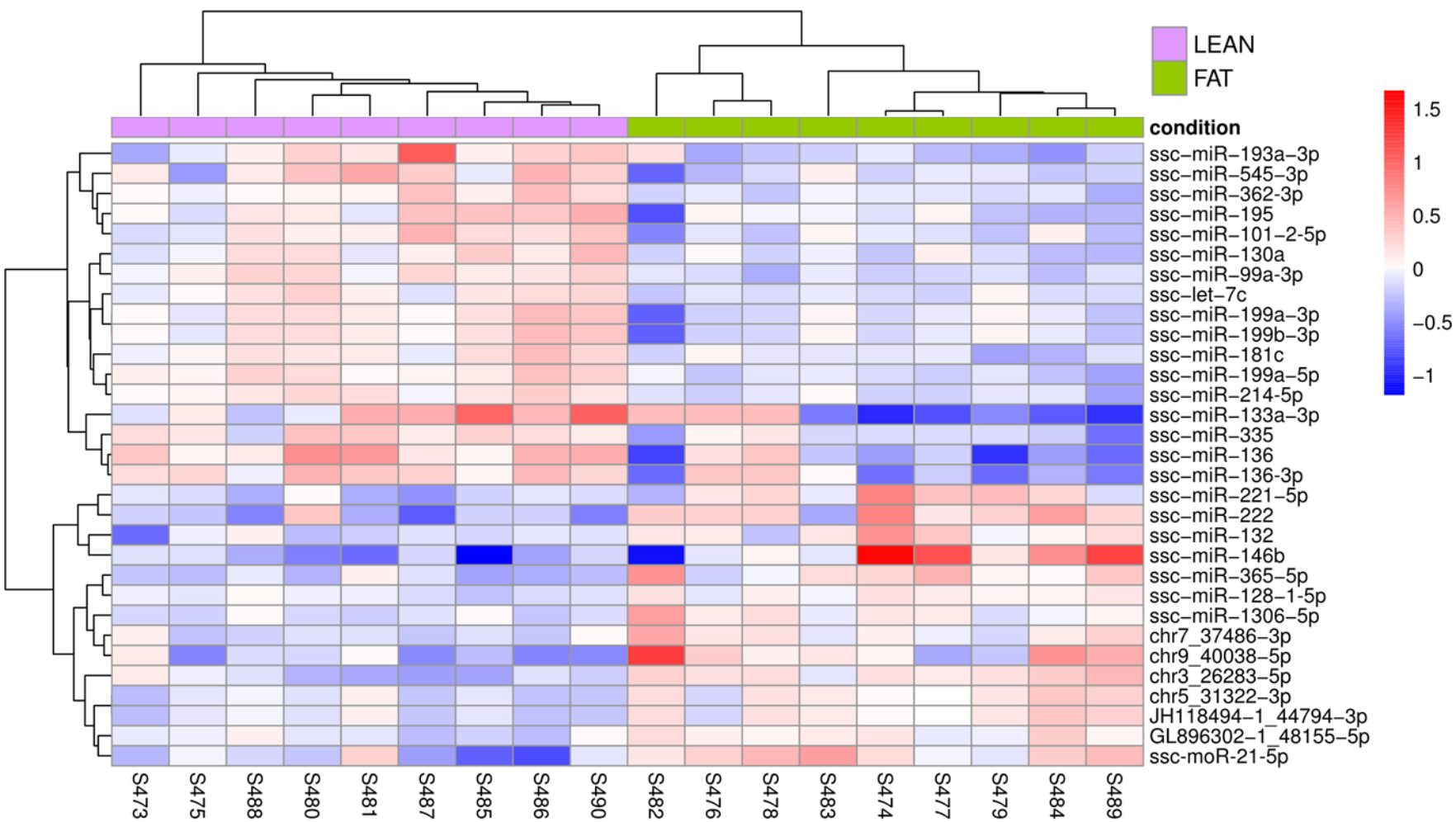
**File: Figure3\_rev.pdf**

## 554 Tables

555 **Table 1.** Short RNAs resulting differentially expressed in backfat of FAT vs LEAN animals  
556 according to RNA-seq data.

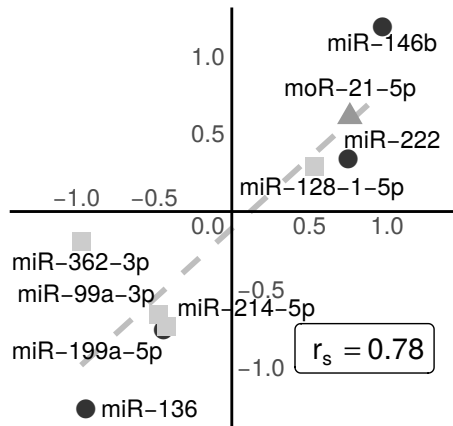
Name	Type	Mature sequence	Average expression (RPM)*	% of expressed sRNAs	Log <sub>2</sub> Fold Change	Adjusted p-value
ssc-miR-146b	known	ugagaacugaaauccauaggc	20131.80	2.13E-02	0.97	0.01
ssc-miR-365-5p	known	gagggacuuucaggggcagcugu	799.48	1.15E-03	0.89	0.00
chr9_40038-5p	new	cuccuggcuggcucgcca	21.81	8.53E-03	0.83	0.01
ssc-miR-221-5p	known	accuggcauacaauagauuuucug	2916.44	1.20E-03	0.77	0.01
ssc-moR-21-5p	moRNA	ctccatggctgtaccacctgtcg	9.63	3.01E-03	0.76	0.01
ssc-miR-222	known	agcuacaucugguacugggucuc	2863.37	2.35E-02	0.75	0.01
chr5_31322-3p	new	ucugagaugugaccugggcau	27.77	6.09E-04	0.72	0.01
JH118494-1_44794-	new	ucugagaugugaccugggcau	11.40	6.09E-04	0.72	0.01
chr7_37486-3p	new	aaguccaucugggucgcc	24.49	6.79E-04	0.69	0.02
chr3_26283-5p	new	uuggcucugcgaggucggcuca	30.91	2.56E-03	0.69	0.00
ssc-miR-128-1-5p	new sister	cggggccgtagcactgtctgag	138.40	5.09E-04	0.53	0.02
ssc-miR-132	known	uaacagucuacagccauggucg	55877.75	4.04E-03	0.50	0.03
ssc-miR-1306-5p	known	ccaccucccugcaaacgucca	70505.29	2.34E-03	0.47	0.02
GL896302-1_48155-	new	ucucuggggccugugucuuaaggcu	12.42	2.09E-03	0.39	0.02
ssc-let-7c	known	ugagguaguagguuguaugguu	2435309.00	5.98E-01	-0.32	0.01
ssc-miR-130a	known	cagugcaauguuuaaagggauc	56716.38	4.26E-02	-0.38	0.02
ssc-miR-181c	known	aacauucaaccugucggugagu	9716.73	1.15E-01	-0.39	0.01
ssc-miR-214-5p	new sister	tgctgtctacactgtctgtgc	93.15	8.13E-03	-0.42	0.00
ssc-miR-199a-5p	known	cccaguguucagacuaccuguuc	5221.81	1.71E-01	-0.44	0.00
ssc-miR-199a-3p	known	acaguagucugcacauugguua	5352.48	1.34E+00	-0.45	0.01
ssc-miR-199b-3p	known	uacaguagucugcacauugguu	5118.61	6.70E-01	-0.45	0.01
ssc-miR-99a-3p	new sister	caagctcgcttctatgggtctg	56.14	1.81E-02	-0.47	0.00
ssc-miR-195	known	uagcagcacagaaauauuggc	6255.99	1.60E-01	-0.50	0.02
ssc-miR-101-2-5p	new sister	tcagttatcacagtgtgatgct	152.31	1.45E-03	-0.61	0.02
ssc-miR-193a-3p	known	aacuggccuacaaagucccagu	6485.75	2.93E-02	-0.62	0.02
ssc-miR-136-3p	new sister	catcatcgtctcaaatgagtct	133.91	8.57E-03	-0.63	0.02
ssc-miR-335	known	ucaagagcaauaacgaaaaaug	1118.26	8.38E-04	-0.75	0.02
ssc-miR-133a-3p	known	uuggucccucaaccagcug	52301.03	1.44E-02	-0.79	0.03
ssc-miR-545-3p	known	aucaacaaacauuuauugugug	381.19	7.45E-04	-0.81	0.04
ssc-miR-136	known	acuccauuuguuuugaugaugga	32337.35	2.42E-03	-0.94	0.01
ssc-miR-362-3p	new sister	aacacacctattcaaggattc	76.01	2.91E-04	-0.97	0.01

557 \* Read Per Million



A

qRT-PCR



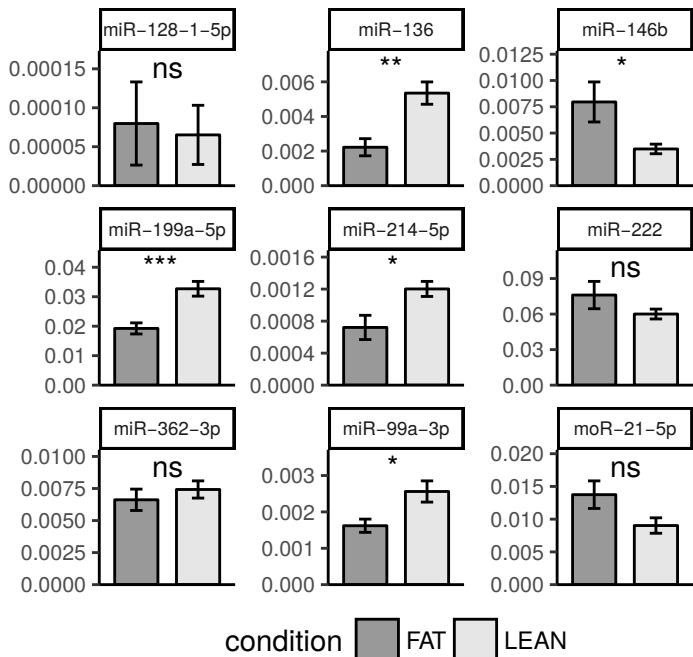
RNA-seq

sRNA category

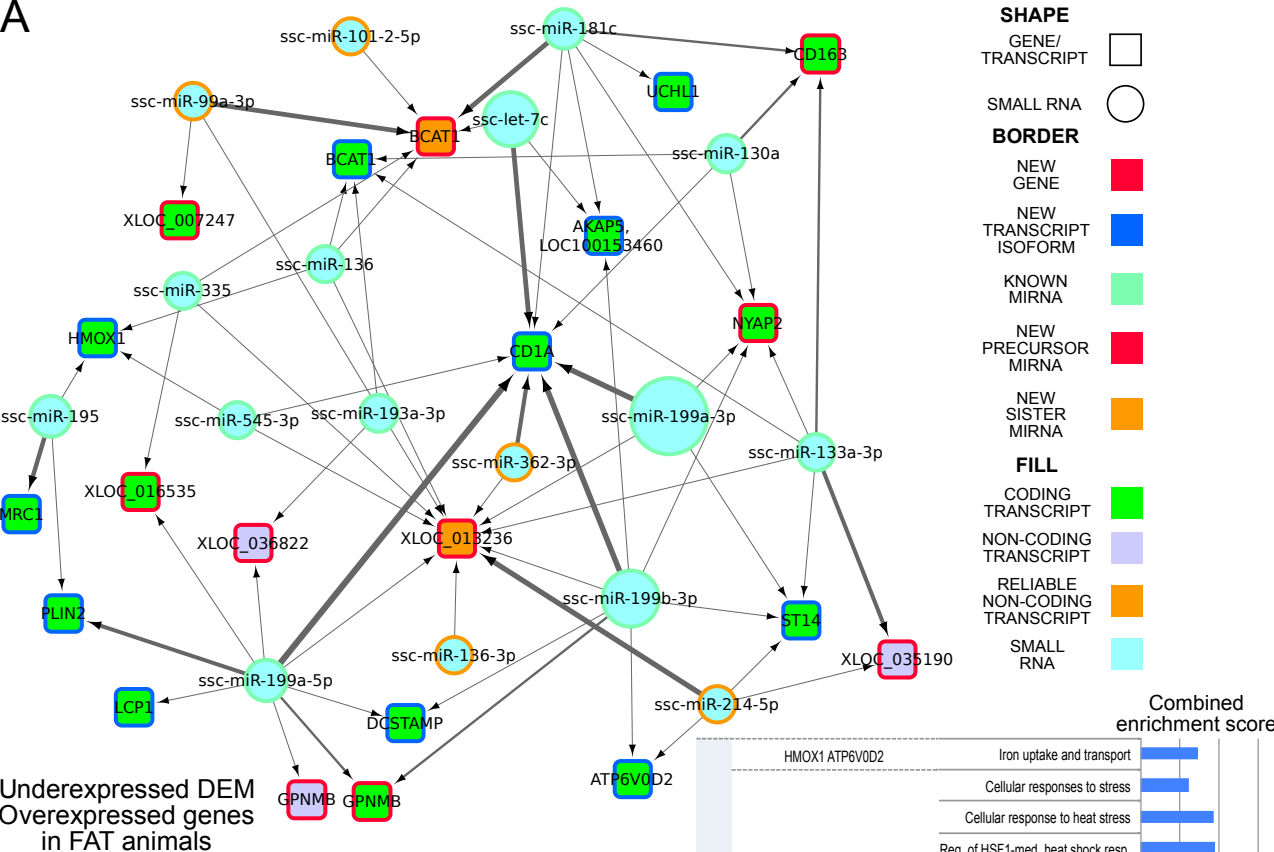
- Known
- ▲ moRNA
- New sister

B

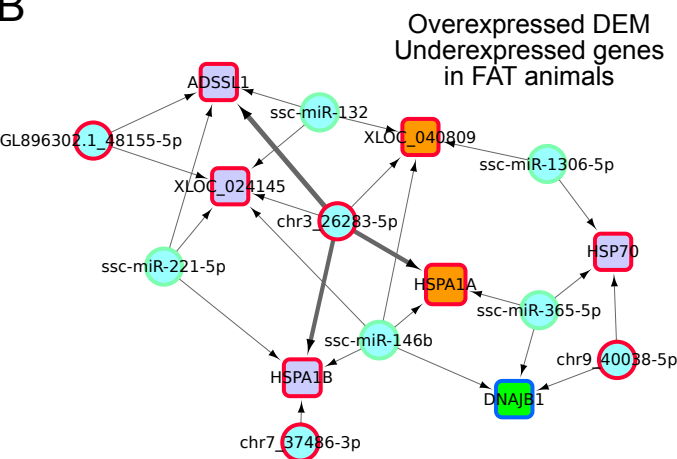
Small RNA qRT-PCR

 $\Delta C_q$ 

A



B



C

